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Retrospective Study of Western Blot Profiles in Immune Sera of Natural Dengue Virus Infections

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The Western blot (WB) assay was used to determine dengue virus antibodies present in human immune sera arising from recent primary and secondary dengue virus infections in Singapore. Cell lysates of dengue-2 virus-infected C6/36 and Vero cells were used. Antibodies directed against structural proteins of dengue-2 virus including envelope (E, gp60/50), capsid-premembrane (C-PrM, gp35), and premembrane (PrM, gp20) were detected, with antibody against envelope protein being most dominant. Similar WB profiles were detected in both primary and secondary dengue virus infections. The reactivity rate of antibodies to dengue-2 virus proteins was higher in infected Vero cell lysate than in infected C6/36 cell lysate, with the exception of antibodies to nonstructural proteins of NS1 and NS3, which were detected predominantly in infected C6/36 cell lysate. More than 75% of ''normal'' individuals (with no complaint of recent dengue virus infection) examined had low levels of dengue virus antibodies, but all presented with similar WB profiles as patients with recent dengue virus infections. This finding reflects a high seroprevalence of dengue virus infections and the long lasting nature of E, C-PrM, and PrM antibodies. Results from this study indicate that in natural dengue virus infections, native E, C-PrM, and PrM antigens of dengue virus are immunogenic and elicit long-lasting antibodies. ***J. Med. Virol. 57:322–330, 1999.***

KEY WORDS: primary and secondary infection; structural proteins; immunogenic

INTRODUCTION

Dengue virus, an arthropod-borne human pathogen, represents a serious public health threat. It is pandemic in the Tropics, and reemerged in the 1990s, causing epidemics in nontropical countries [Halstead, 1990; Monath, 1994]. Clinical manifestations of dengue virus infection range in severity from simple febrile illness to haemorrhagic fever and a potentially fatal haemorrhagic shock syndrome [Halstead, 1988, 1989]. There is no specific treatment for this infection.

Antibodies derived from the various subunits or recombinant dengue virus proteins offered different degrees of protection in animal studies [Schlesinger et al., 1987; Zhang et al., 1988; Kaufman et al., 1989; Falgout et al., 1990; Bray and Lai, 1991; Putnak et al., 1991; Srivastava et al., 1995; Staropoli et al., 1997]. The various subunit dengue virus proteins include virus premembrane, envelope, and nonstructural protein NS1. However, there is no current consensus of opinion on the most suitable type of vaccine.

To date, the most promising vaccine has been live or attenuated whole virus, which is used as mono-, bi-, tri-, or tetravalent vaccine. These candidate vaccines yielded encouraging results in clinical trials, showing strong immunogenicity with no untoward clinical signs and symptoms in adult volunteers or only minimal clinical manifestation in children [Bhamarapravati and Yoksan, 1997]. Phenotypic reversion of attenuated viruses to parental viruses was not evident [Khin et al., 1994; Bhamarapravati and Yoksan, 1997] and thus suggests the safety of attenuated virus vaccine, though the risk of virus reversion could not be totally excluded. This study aimed to explore the possible neutralizing antibodies derived from whole virus vaccination, through in vivo insight of humoral immune response to native dengue virus antigens in natural dengue viral infections. This method would help to identify the virus protein components that were B-cell immunogenic, that elicited neutralising antibodies, and thus would protect from dengue virus infection.

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MATERIALS AND METHODS

Clinical Samples

Blood specimens were selected from 520 hospital attendees, (460 patients seeking medical treatment, and laboratory samples of 60 normal healthy individuals for routine health screening). Four hundred twenty-seven of the 460 patients demonstrated symptoms of viral fever and were diagnosed clinically as having dengue virus infection. Two specimens (first blood and second blood) taken at an interval of 2 weeks were collected from each of these patients. Ideally, the first blood was collected in the acute phase of infection, and second blood in the convalescent phase. However, some of the patients attended the hospital only in the late stage of convalescence, and thus, the first blood was obtained late in convalescence and the second in the recovery stage, when antibodies levels had fallen. A single specimen was obtained from 30 other patients hospitalised for illnesses other than viral infection, giving the total number of samples collected as 944 (854 from fever patients, 30 from hospitalised patients, and 60 from normal healthy individuals).

Dengue Virus Antigens

All dengue virus antigens were purchased from the Virus Research Institute, Thailand. Dengue virus type 1 (Hawaii strain) and type 2 (Trl 751) were used for haemagglutination inhibition assay (HI), diluted to a concentration of 8 haemagglutination units (HA) using 10% proteose peptone in boric buffer saline (BBS) at pH 9.0 (0.12 M NaCl, 0.05 M boric acid, and 0.02 N NaOH); and a cocktail of antigens of dengue type 1, 2, 3, and 4 (Venture Technologies Sdn Bhd, Malaysia) were used for IgM capture enzyme-linked immunosorbent assay (ELISA), at a dilution of 1:2.

Adsorption of Sera for HI

Preadsorption of serum sample was undertaken to reduce nonspecific interference. One hundred microliters of sera were added to 400 µl of BBS, followed by 500 µl of 25% acid-washed kaolin suspension (Sigma, St. Louis, MO), mixed and incubated for 30 min at room temperature (RT), centrifuged for 10 min at 2,000 rpm and 50 µl of packed goose red blood cells was added. The cells were then mixed, left on ice for 1 hr, then centrifuged for another 10 min at 2,000 rpm. The supernatant collected was used as a 1:10 dilution serum sample.

HI

Adsorbed serum was diluted two-fold from 1:10 to 1:10,240 in 10% proteose peptone in BBS, pH 9.0 in a V-bottomed 96-well plate, leaving 25 µl of diluted serum in each well. Twenty-five microliters (8 HA unit) of dengue virus antigens were added to each well, and the plate then left for 2 hr at RT. Fifty microliters of 0.32% goose red blood cells in phosphate buffer (0.15 M NaCl, 0.044 M Na_2HPO_4, and 0.156 M NaH_2PO_4) at pH 6.0 for dengue-1 and pH 6.2 for dengue-2 were added, and the cells allowed to settle at RT for 1 hr, after which endpoint titre for HI of the sample was read.

IgM Capture ELISA

A 96-well plate was coated with 100 µl rabbit anti-human IgM (1:1,000 Dako coating buffer) overnight at 4°C. The plate was washed three times with phosphate-buffered saline (PBS)-Tween (PBS, pH 7.6, 0.05% Tween-20), blocked with 200 µl PBS containing 10% fetal calf serum, and left to incubate for 2 hr at RT. The plate was washed as described earlier, and then kept dry at 4°C until use. Patient serum diluted 1:100 with PBS containing 1% bovine serum albumin (BSA) and 100 µl was dispensed into each well, in duplicate, incubated at RT for 2 hr, and washed five times with PBS-Tween with a 1-min soaking time between washes. One hundred microliters of dengue antigen cocktail or antigen control were added to the wells and left overnight at 4°C. On the following day, the plate was washed as described earlier, and 100 µl of 1:2 diluted flavivirus monoclonal antibody (obtained from ATCC hybridoma: 4G2) added. The plate was incubated for 1 hr at RT, and the washing steps repeated. One hundred µl of 1:1000 rabbit anti-mouse IgG-HRP conjugate was added and incubated for 1 hr at RT. The plate was washed, and 100 µl of substrate (10 mg of *o*-phenylene diamine, 5 µl 30% hydrogen peroxide in 25 ml of 0.05M phosphate-citrate buffer pH 5.0) was added for colour development in the dark for 30 min at RT. The colour reaction was stopped with 50 µl 2.5 M sulphuric acid, and the results read at an optical density of 492 nm/620 nm.

Western Blot

Two cell lines, mosquito cells (C6/36) and mammalian cells (Vero), were used for dengue virus antigen preparation. The cell lines were infected with New Guinea C dengue type 2 virus at a multiplication of infection (m.o.i.) of 10. The infected cells were incubated at 28°C for 2 days (C6/36) or 37°C for 4 days (Vero). After the incubation period, cells were lysed with 2% sodium dodecyl sulphate (SDS), and the lysates were denatured by boiling for 5 min. Denatured dengue virus proteins were electrophoresed in a 12.5% polyacrylamide gel, and blotted onto a nitrocellulose membrane, 0.45 µm pore size (Bio-Rad Laboratories, USA). The membrane was cut into strips and blocked with 5% skim milk in PBS for 45 min at RT. The blocking buffer was washed three times using PBS-Tween 20, with an interval of 10 min between the washes. Serum samples were diluted 1:100 or 1:1,000 (depending on the HI titre) with blotting buffer (5% skim milk, 4% goat serum in Tris buffer). Five hundred microliters of the diluted serum were added to each strip, and incubated overnight at RT. On the following day, the strip was washed as described above, and 500 µl of 1:2,000 diluted goat anti-human IgG alkaline phosphatase conjugate were added, and allowed to incubate for 30 min at RT. The washing steps were repeated. Five hundred microliters of substrate solution of 5-bromo-4-

chloro-3-indolyl-phophate and nitroblue tetrazolium (Genelabs Diagnostic, Singapore) were added for 10 min to allow for colour development at RT. The strip was washed twice with deionised water, with a 5-min interval between washes.

RESULTS

Dengue Virus Infected, Symptomatic (Viral Fever) Patients

(a) HI and IgM capture assay. Two samples, of approximately 2-week intervals obtained from 427 patients with symptoms of viral fever, were screened using HI assay. The following criteria were used to differentiate patients with recent and past dengue virus infections. (i) Recent infection if ⩾ four-fold increase in HI titre of dengue virus antibody; and/or (ii) a high titre (⩾2,560) of dengue virus antibody found in any one sample, and/or (iii) the presence of IgM antibody, as detected by IgM capture ELISA. Three hundred twenty-six patients satisfied the criteria for recent dengue virus infection. The rest (101 patients) demonstrated a persistently lower level of antibodies (HI ⩽ 160), and were assumed to have had a past dengue infection.

Patients infected recently were subclassified further as having primary or secondary antibody response (i.e., response to an initial infection or reinfection). A primary serologicla response was defined in patient with a negative (HI < 10), low (HI = 10–80), or moderate dengue virus antibody (HI = 160–1280) in the first blood specimen, and a titre of 40 or greater, but less than 2,560 in the second blood specimen. Absence or presence of antibody in the first blood specimen, with a fourfold or greater rise in titres to 2560 or higher in the second blood specimen was considered as a secondary type of response. The explanation for patients who demonstrated a falling HI titre could have been due to their visiting the hospital after the acute phase of infection. The first blood specimen of these patients was likely to have been obtained at the peak of convalescent phase, and the second specimen collected at a later stage of convalescence when the antibody was falling.

Using the above criteria and a HI titre of 2,560 as cutoff, 170 patients were classified with primary dengue virus infection, and 156 patients with secondary infections. More than half of the first blood specimens (67.1%) from the primary infected patients showed negative results in HI assay, 23.5% tested had low titres of dengue virus antibody, and 9.4% had moderate levels of dengue virus antibody. Among these with primary infections, 59.4% demonstrated low HI titres in their second blood specimens, and 40.6%, moderate HI titres. In contrast, only 10.3% of the first blood specimen from patients with secondary response were negative in the HI assay. Approximately 19.9% of them showed low HI titres, 30% and 39.7% with moderate or high HI titres, respectively. All except eight of the patients with secondary dengue virus infection showed high HI titres of dengue virus antibodies in their second blood specimens. Details of the results are shown in Table I. IgM capture-ELISA was performed on the first blood specimen of all patients and IgM antibodies to dengue virus was detected in 74.1% (126/170) of the patients with primary antibody response, 64.1% (100/156) in patients with secondary antibody response, and none from patients with past dengue virus infections.

TABLE I. Haemagglutination Inhibition Assay (HI) Results for Patients With Recent Dengue Virus Infection

	Sample number	
HI titer	1st bleed	2nd bleed
Primary responses		
Neg (<10)	114	0
Low (10–80)	40	101
Moderate (160–1280)	16	69
Subtotal	170	170
Secondary responses		
Neg (<10)	16	0
Low (10–80)	31	4
Moderate (160–1280)	47	4
High (⩾ 2560)	62	148
Subtotal	156	156
Total	426	426

(b) Western blot (WB). The Western blot technique (WB) was used to examine the pattern of antibody development towards different protein components of dengue virus in patients. The antigen source used in WB was derived from dengue-2 virus-infected C6/36 (henceforth, termed as D2-C6/36 WB) or dengue 2 virus-infected Vero (termed as D2-Vero WB) cell lysates.

Monoclonal antibody (MAb) specific to envelope (E) protein of dengue-2 virus (obtained from ATCC hybridoma: HB46 3H5-1) was used to confirm the position of E protein bands in the WB. Figure 1 shows the E MAb reactive bands on the WB strips. The E antigens comprised two broad diffuse bands in both D2-C6/36 and D2-Vero WBs. The larger is a protein band with a molecular weight of 60 kDa, termed as Em (major envelope protein), and a smaller envelope protein of M_r 50 kDa is designated as Es (small envelope protein). Besides these two bands, an additional band with a higher M_r, estimated to be 67 kDa, was also observed in the D2-Vero but not D2-C6/36 WB. This additional protein band may represent a different glycosylated form of virus E protein and has been designated as El (large envelope protein).

Due to the lack of MAb to other dengue viral specific proteins in this study, dengue viral proteins other than E protein were designated according to their molecular weights [Churdboonchart et al., 1991]. Protein with M_r 97 kDa was assigned as nonstructural NS5, M_r 67 kDa protein as nonstructural NS3, and M_r 46 kDa protein as nonstructural NS1. Although both NS3 and El were proteins of M_r 67 kDa, El appeared as a diffused band (as it is glycosylated) whilst the nonglycosylated NS3 protein presented as a defined band. Proteins with a lower molecular weight, such as M_r 35, were referred to

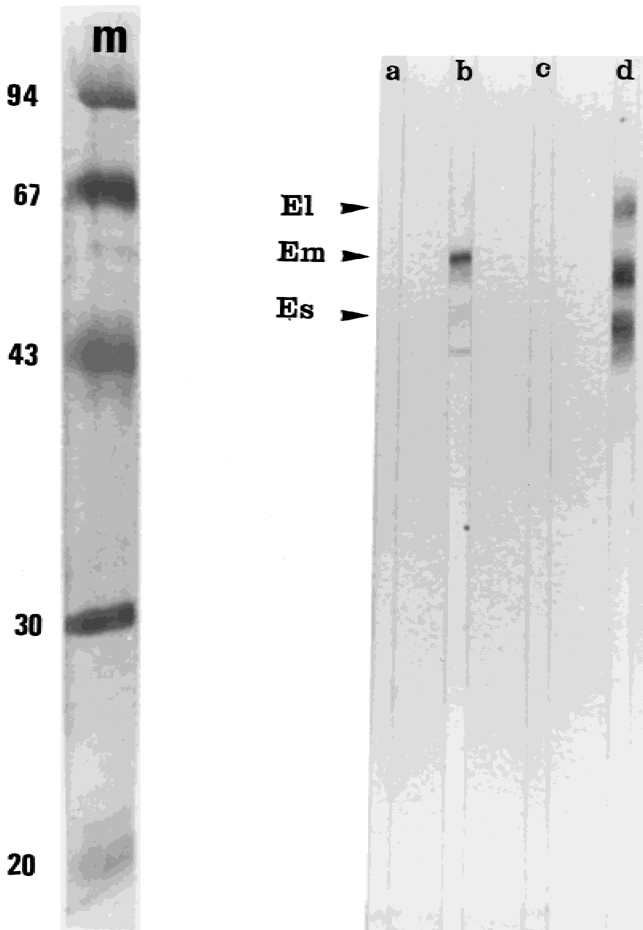

Fig. 1. Reactivity of monoclonal antibody specific for dengue-2 virus envelope protein (HB46 3H5-1) to mock-infected C6/36 cell lysate (a), dengue-2 virus-infected C6/36 cell lysate (b), mock-infected Vero cell lysate (c), and dengue-2 virus-infected Vero cell lysate Western blot strip (d). El, large envelope protein; Em, major envelope protein; Es, small envelope protein; m, corresponding molecular weight markers in kilodaltons.

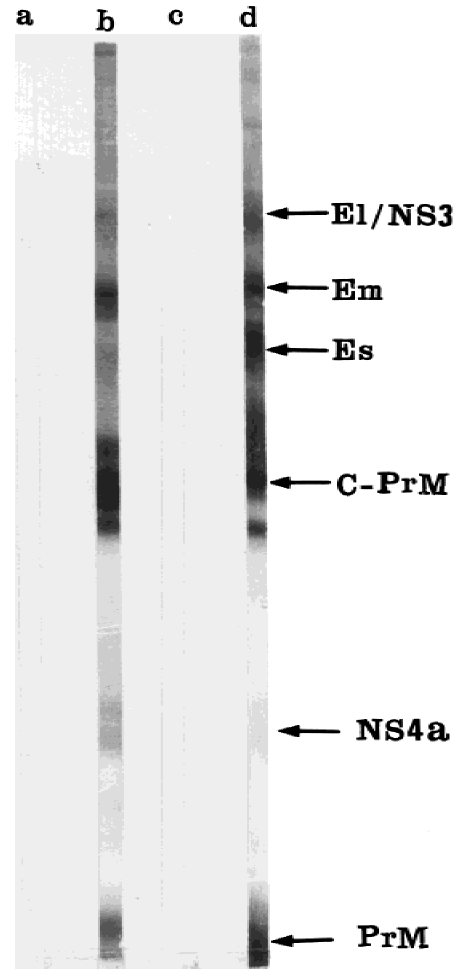

Fig. 2. Typical Western blot profiles of patient positive with dengue virus antibody. Dengue virus antibody reactivity to mock-infected C6/36 cell lysate (a), dengue-2 virus-infected C6/36 cell lysate (b), mock-infected Vero cell lysate (c), and dengue-2 virus-infected Vero cell lysate (d). El, large envelope protein; NS3, nonstructural protein 3; Em, major envelope protein; Es, small envelope protein; C-PrM, capsid-premembrane protein; NS4a, nonstructural protein 4a; PrM, premembrane protein.

as uncleaved capsid-premembrane (C-PrM), M_r 28 kDa as nonstructural NS4a, and M_r 20 kDa as premembrane (PrM). Internal controls were added to prevent misinterpretation of antibody reacting to host cell proteins (C6/36 and Vero cells) with the same molecular weight as dengue virus-specific proteins. These controls involved the testing of patients' sera with WB strips derived from mock-infected C6/36 or Vero cell lysate in parallel with dengue-2 virus-infected cell lysate WB strips. Figure 2 shows typical WB reaction bands for a dengue virus antibody-positive sample. Most of the visible bands were specific to dengue-2 virus proteins, appeared only in the infected cell lysate strips, and were not present in the corresponding strips (mock-infected cell lysate).

Western blot was carried out on 320 second blood specimens of patients with recent dengue virus infection. One hundred sixty-four specimens were from patients with primary dengue virus infection and 156 from patients with secondary infection.

(c) Antibodies detected using WB derived from dengue-2 virus infected-C6/36 cell lysate. Figure 3 shows the antibody response of patients with primary and secondary dengue virus infection with respect to dengue-2 virus-specific antigens in the D2-C6/36 WB. Antibodies against both structural (Em, Es, C-PrM, PrM) and nonstructural proteins (NS1, NS3, and NS4a) were detected. The antibody reactivity rates (% rate) to structural proteins were higher than nonstructural proteins. The highest percentage rate was detected for antibodies to dengue-2 virus envelope proteins, particularly anti-Em. More than 90% of the patients with primary dengue virus infection were detected with antibody to Em, whereas the detection rate for patients with secondary infection was 100%. In primary dengue virus infection, percentage rate of antibodies to PrM and C-PrM was found to be 48 and 76%, respectively, and these percentages were increased to

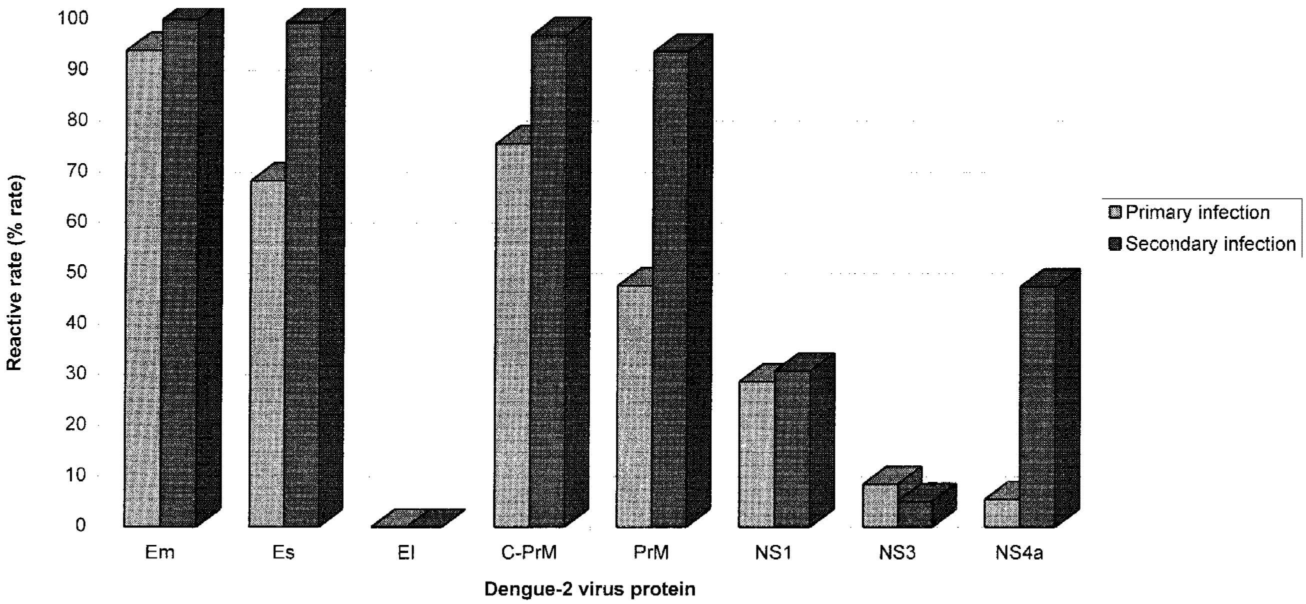

Fig. 3. Antibody reactivity to C6/36 cell-derived dengue-2 virus protein in patients with recent primary and secondary dengue virus infection.

more than 90% in patients with secondary infection. Detection of antibodies to dengue-2 virus nonstructural proteins was less prevalent. Only approximately 30% of the dengue virus-infected patients showed the presence of antibody to NS1, and fewer than 10% for antibody to NS3 protein. Although antibody to NS4a was lowest in primary infection, the percentage rate was increased to almost 50% in patients with secondary dengue virus infection. Percentage rates of antibodies to all dengue-2 virus proteins, except NS3 protein, were higher in patients with secondary dengue virus infection

(d) Antibodies detected using WB derived from dengue-2 virus infected-Vero cell lysate. Compared with D2-C6/36 cell lysate WB, D2-Vero cell lysate-derived WB did not detect antibody to NS3, but did detect antibodies to El (the large envelope protein), unlike its counterpart (D2-C6/36 WB). Figure 4 shows the D2-Vero WB results for patients with recent dengue virus infection. Antibodies to Em, Es, El, C-PrM, PrM, NS1, and NS4a were detected. Antibody to Em appeared to be most prevalent, followed closely by anti-Es or anti-C-PrM, anti-PrM, anti-NS4a, and anti-El. The antibody percentage rate for Em and Es was approximately 90% for primary infected patients, but 100% for patients with secondary dengue virus infection. Antibody percentage rate to NS1 protein was low and detectable only in less than 2% of patients with secondary infection. Antibody percentage rates for all detectable dengue virus-specific proteins were generally higher for patients with secondary infection.

The overall trends for antibody percentage rates to virus-specific protein bands detected with the D2-C6/36 and D2-Vero WBs were fairly similar. The D2-Vero WB however, gave higher detection rates for antibodies to E (Em and Es), C-PrM, and NS4a, whereas antibodies against NS1 and NS3 were detectable more readily by the D2-C6/36 WB.

Patients With Past Dengue Virus Infection, Hospitalised Patients (for Non-Dengue Virus-Related Illnesses) and Normal Healthy Individuals

By studying the WB profiles of patients with past dengue virus infection (PDI), an attempt was made to identify long-lasting antibodies. HI assay showed that all patients with past infections maintained a low-to-moderate level of antibodies, with the majority (87/101 = 86.1%) having low, and the minority (14/101 = 13.9%), a moderate antibody level. Figures 5 and 6 show the prevalence rate of antibodies to different dengue-2 virus-specific protein components detected by WBs. Antibody to envelope protein (Em [100%] and Es [85–96%]) appeared to be most prominent, followed by antibodies to C-PrM (85–98%) or PrM (67–79%). This finding was evident in both the D2-C6/36 and D2-Vero WB, but with higher antibody percentage rate towards the later. Moderate-to-low prevalence of antibodies to NS1 (38%), NS3 (21%), and NS4a (6%) were also detected in the D2-C6/36 WB. The D2-Vero WB, however, showed a low antibody percentage rate only to NS4a (8%). These results suggest that all patients with past dengue virus infection retain antibody towards the virus envelope protein (Em), but not all patients sustain antibodies against C-PrM, PrM proteins nor the non-structural proteins.

More than 75% of the persons selected at random from hospitalised patients (HP) and normal healthy individuals (NHI) screened positive for dengue-1 and -2 virus antibodies (by HI assay). The antibody percentage rate was 80% (24/30) for hospitalised patients and 78% (47/60) for NHI. Of these, 83% (20/24) of immune

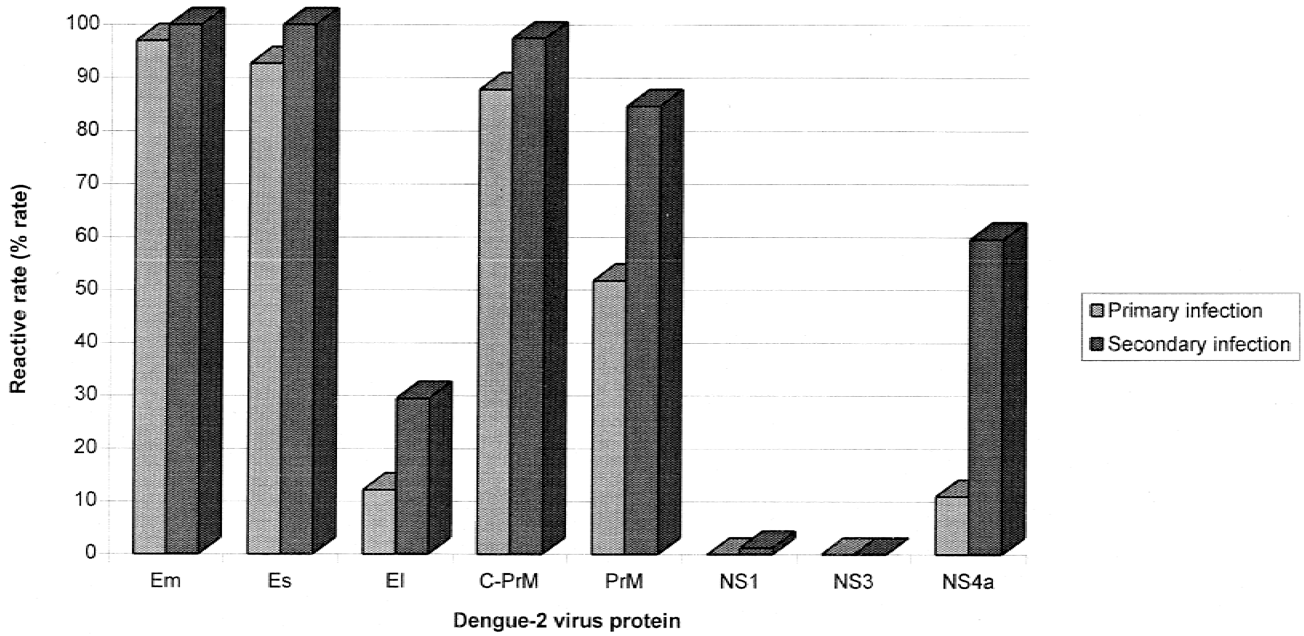

Fig. 4. Antibody reactivity to Vero cell-derived dengue-2 virus protein in patients with recent primary and secondary dengue virus infection.

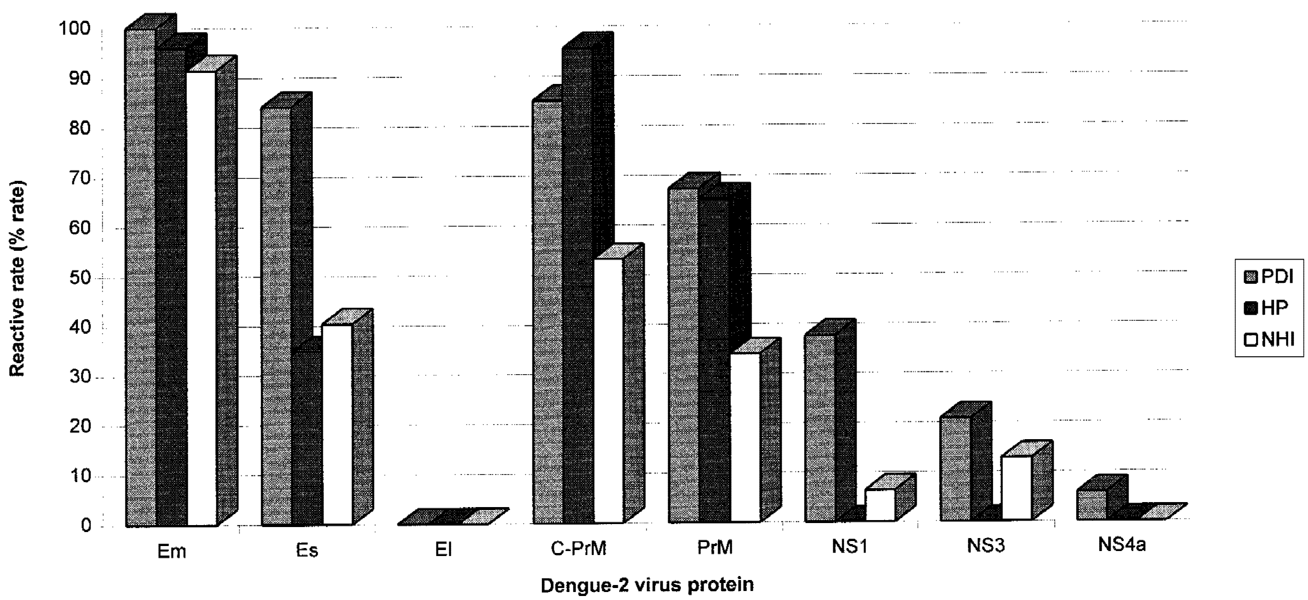

Fig. 5. Antibody reactivity to C6/36 cell-derived dengue-2 virus protein in patients with past dengue virus infection and demonstrating viral fever (PDI), hospitalised patients (HP), and normal healthy individuals (NHI).

hospitalised patients maintained low levels of dengue antibodies, and 13% (3/24) showed moderate antibody levels, with one sample giving high HI titres. A similar proportion, 87% (41/47), of the NHI retained a low level of dengue virus antibodies, 13% (6/47), a moderate antibody level. These findings were in parallel with results obtained for patients with past dengue virus infection. For HP, reactivities towards all four structural proteins, Em, Es, C-PrM, and PrM, of the virus were observed. Compared with D2-Vero WB, D2-C6/36 WB showed high antibody percentage rate to Em and C-PrM, but relatively lower antibody reactivity rate to Es and PrM. Antibodies to El, NS4a, NS1, or NS3 were not detected.

More than 90% of NHI showed antibody reactivities to Em in both the D2-C6/36 and D2-Vero WB. High percentage rates (80–95%) of antibody to C-PrM and Es were also seen in the D2-Vero WB. The antibody percentage rates for the same proteins were lower, 53% for C-PrM and 40% for Es as detected by D2-C6/36 WB. Antibody to El (19%) was detected using the D2-Vero WB but not D2-C6/36 WB, whereas antibodies to den-

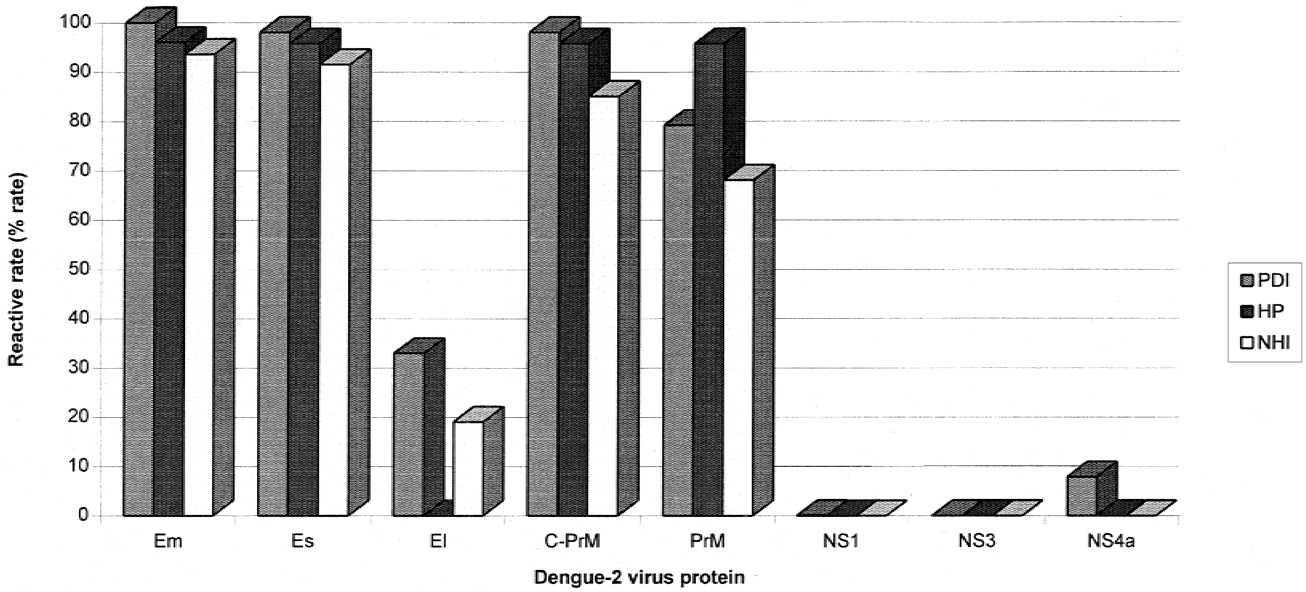

Fig. 6. Antibody reactivity to Vero cell-derived dengue-2 virus protein in patients with past dengue virus infection and demonstrating viral fever (PDI), hospitalised patients (HP), and normal healthy individuals (NHI).

gue-2 virus-specific nonstructural proteins (NS1 and NS3) were only detected in low percentages in the D2-C6/36 WB.

Data obtained from these three groups of individuals (or patients) illustrate concomitantly the long-lasting nature of antibodies to structural proteins of dengue-2 virus.

DISCUSSION

To our knowledge, this is the first large-scale retrospective, seroepidemiological study using Western blot as a means to investigate the antibody status of humans exposed to natural primary and secondary dengue virus infections. An advantage of WB is the ability to characterise the antibodies and differentiate antibodies of structural and nonstructural protein origin. The results obtained from this study demonstrated consistently that dengue virus infections resulted regularly in development of antibodies to virus structural proteins. These are envelope (Em, Es or El), capsid-premembrane (C-PrM), and premembrane (PrM) proteins expressed in dengue-2 virus-infected cells. These same proteins, in particular the envelope protein, also appeared to elicit high antibody responses, and were found to be sustained in individuals with no sign of recent dengue virus infections. Because clinical histories of dengue virus infections among these individuals were not available, it was not possible to determine the time of infection, and thus, the retention period of these antibodies.

However, the detection of antibodies to these dengue-2 virus structural proteins among past dengue virus-infected patients as well as the other individuals studied indicate that they could be sustained for quite some time after infection. The study of Vaughn et al. [1996] showed that low levels of dengue-2 virus neutralising antibodies were still detected 2 years post-dengue virus (live-attenuated) immunisation in volunteers. Persistence of neutralising antibodies for more than four decades has been reported in some individuals in Japan and Greece, suggesting homotypic immunity to dengue virus is life-long [Halstead, 1974; Papaevangelou and Halstead, 1977; Tadano et al., 1983]. The predominant proteins present in purified dengue-2 virus were E, PrM, and a small amount of C. Antibodies generated from these proteins were believed to contribute to partial protection in animal models [Putnak et al., 1996]. Passive protection of mice from homotypic and heterotypic dengue viruses have been demonstrated using monoclonal antibodies directed to E-glycoprotein and pre-M-glycoprotein [Kaufman et al., 1987, 1989]. All these results support the notion that antibodies to dengue-2 virus structural proteins (E, PrM, and probably C-PrM) possess the characteristic of viral neutralisation. Antibodies to E, PrM/M, and C-PrM when acting alone or in combination were shown to confer different degrees of protective immunity in animals from homotypic dengue virus infection [Kaufman et al., 1989; Bray and Lai, 1991; Putnak et al., 1991; Staropoli et al., 1997]. The combination, however, proved to be more effective than when they were used singly [Bray and Lai, 1991]. Furthermore, Bray and colleagues [1996] demonstrated that C-PrM-E or PrM-E portions of a chimeric dengue-2 /3 viruses elicited protective immunity to homotypic virus challenge.

The NS1 protein of dengue virus was also present as a potential candidate antigen for eliciting antibody response. Antibody response to NS1 was detected in both

primary and secondary infections, as detected by WB using antigens from dengue-2 virus-infected mosquito cell line, but not from those derived from an infected mammalian cell line. However, despite the fact that NS1 is present in abundance on surfaces of dengue-2 virus-infected cells [Hase et al., 1987] and has been shown to confer protective immunity to homotypic dengue virus [Schlesinger et al., 1987; Zhang et al., 1988; Fagout et al., 1990; Srivastava et al., 1995], results from this study do not indicate NS1 to be as immunogenic as the other structural proteins of dengue-2 virus. Only in approximately one-third of patients with primary or secondary dengue virus infections were antibody to NS1 detected, versus an almost 100% response rate to envelope (Em and Es), C-prM, or prM proteins. In addition, the percentage rate of detectable anti-NS1 in individuals with past dengue virus infections was also minimal. This finding may suggest that NS1 is less immunogenic and has lesser importance as a long-lasting antibody. However, WBs used in this study may have been less sensitive in detecting NS1 antibody. This possibility may have existed because denatured proteins containing monomeric NS1, which has been shown to be less immunogenic than the native dimeric form of NS1 protein [Falconar and Young, 1991], were used in the assays.

Antibodies to the other two nonstructural proteins, NS3 and NS4a, though present, were relatively low in prevalence. They are thus less important in eliciting antibody, and the antibodies elicited are not as long lasting in comparison with antibodies derived from dengue-2 virus-specific structural proteins. The NS3 protein, however, contains multiple T-cell epitopes and is an important target for T_c-cells [Kurane et al., 1991, 1993]. Hence, NS3 protein may be less immunodominant for B-cells, though it is immunogenic for dominant cytotoxic T lymphocytes (CTL) and helper T-cell response.

Although Churdboonchart et al. [1991] showed that antibody to NS5 could be a candidate marker for recent dengue virus infection, we did not detect the presence of anti-NS5 in any of the samples from patients with past or recent dengue virus infections.

Human convalescent sera, or polyclonal immune sera, offered the possibilities of analysing specificities of antibody population to dengue virus antigen and could aid in pin-pointing immunologically active sites of dengue virus during a natural infection and their corresponding key ingredients in protective immune response. In this study, it was concluded that structural proteins were potential components for dengue virus vaccine, with E protein presenting as the most promising candidate.

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REFERENCES

Bhamarapravati N, Yoksan S. 1997. Live attenuated tetravalent dengue vaccine. In: Gubler DJ, Kuno G, editors. Dengue and dengue hemorrhagic fever. CAB International. p 367–377. Wallingford, UK, Madison Ave, NY.

Bray M, Lai CJ. 1991. Dengue virus premembrane and membrane proteins elicit a protective immune response. Virology 185:505–508.

Bray M, Men R, Lai CJ. 1996. Monkey immunized with intertypic chimeric dengue viruses are protected against wild-type virus challenge. J Virol 70:4162–4166.

Churdboonchart V, Bhamaraprati N, Peampramprecha S, Sirinavin S. 1991. Antibodies against dengue viral proteins in primary and secondary dengue hemorrhagic fever. Am J Trop Med Hyg 44:481–493.

Falconar AKI, Young PR. 1991. Production of dimer-specific and dengue virus group cross-reactive mouse monoclonal antibodies to the dengue 2 virus non-structural glycoprotein NS1. J Gen Virol 72: 961–965.

Falgout B, Bray M, Schlesinger JJ, Lai CJ. 1990. Immunization of mice with recombinant vaccinia virus expressing authentic dengue virus nonstructural protein NS1 protects against lethal dengue virus encephalitis. J Virol 64:4356–4363.

Halstead SB. 1974. Etiologies of the experimental dengues of Silver and Simmons. Am J Trop Med Hyg 223:974–982.

Halstead SB. 1988. Pathogenesis of dengue: challenges to molecular biology. Science 239:476–486.

Halstead SB. 1989. Antibody, macrophages, dengue virus infection, shock, and hemorrhage: a pathogenic cascade. Rev Infect Dis 11:S830–S839.

Halstead SB. 1990. Global epidemiology of dengue hemorrhagic fever. Southeast Asian J Trop Med Public Health 21:636–641.

Hase T, Summers PL, Eckels KH, Baze WB. 1987. An electron and immunoelectron microscopic study of dengue-2 virus infection of cultured mosquito maturation events. Arch Virol 92:273–291.

Kaufman BM, Summers OL, Dubois DR and Eckels KH. 1987. Monoclonal antibodies against dengue 2 virus E-glycoprotein protect mice against lethal dengue infection. Am J Trop Med Hyg 36:427–434.

Kaufman BM, Summers PL, Dubois DR, Houston Cohen DR, Gentry MK, Timchak RL, Burke D, Eckels KH. 1989. Monoclonal antibodies for dengue virus prM glycoprotein protect mice against lethal dengue infection. Am J Trop Med Hyg 41:576–580.

Khin MM, Jirakanjanakit N, Yoksan SM Bhamarapravati N. 1994. Infection, dissemination, transmission and biological attributes of DEN 2, PDK 53 candidate vaccine virus after oral infection in Aedes aegypti. Am J Trop Med Hyg 51:864–869.

Kurane I, Brinton MA, Samson AL, Ennis FA. 1991. Dengue virus-specific, human CD4+ CD8- cytotoxic T-cell clones: multiple patterns of virus-cross reactivity recognized by NS3-specific T-cell clones. J Virol 65:1823–1828.

Kurane I, Dai L-C, Livingstone PG, Reed E, Ennis FA. 1993. Definition of an HLA-Dpw2-restricted epitope on NS3, recognized by a dengue virus serotype-cross-reactive human CD4+ CD8– cytotoxic T-cell clone. J Virol 67:6285–6288.

Monath TP. 1994. Dengue: the risks to developed and developing countries. Proc Natl Acad Sci USA. 91:2395–2400.

Papaevangelou G, Halstead SB. 1977. Infections with two dengue viruses in Greece in the 20th century. Did dengue hemorrhagic fever occur in the 1928 epidemic? J Trop Med 80:46–51

Putnak A, Feighny R, Burrous J, Cochran M, Hackett C, Smith G, Hoke C. 1991. Dengue-1 virus envelope glycoprotein gene expressed in recombinant baculovirus elicits virus-neutralizing antibody in mice and protects them from virus challenge. Am J Trop Med Hyg 45:159–167.

Putnak R, Cassidy K, Conforti N, Lee R, Sollazzo D, Truong T, Ing E, Dubois D, Sparkuhl J, Gastle W, Hoke C. 1996. Immunogenic and protective response in mice immunized with a purified, inactivated, dengue-2 virus vaccine prototype made in fetal rhesus lung cells. Am J Trop Med Hyg 55:504–510.

Schlesinger JJ, Brandriss MW, Walsh EE. 1987. Protection of mice against dengue 2 virus encephalitis by immunization with the dengue 2 virus non-structural glycoprotein NS1. J Gen Virol 68: 853–857.

Srivastava AK, Putnak JR, Warren RL, Hoke Jr CH. 1995. Mice im-

munized with a dengue type 2 virus E and NS1 fusion protein made in Escherichia coli are protected against lethal dengue virus infection. Vaccine 13:1251–1258.

Staropoli I, Frenkiel M-P, Megret F, Deubel V. 1997. Affinity-purified dengue-2 envelope glycoprotein induces neutralizing antibodies and protective immunity in mice. Vaccine 15:1946–1954.

Tadano M,Okuno Y, Fukunaga T, Fukai K. 1983. Retrospective serological studies on dengue epidemics in Osaka and Okinawa. Biken J 26:165–167.

Vaughn DW, Hoke Jr CH, Yoksan S, LaChance R, Innis BL, Rice RM, Bhamarapravati N. 1996. Testing of a dengue 2 live-attenuated vaccine (strain 16681 PDK 53) in the American volunteers. Vaccine 14:329–336.

Zhang YM, Hayes E, McCarthy T, Dubois D, Summers P, Eckels K, Chanock RM, Lai CJ. 1988. Immunization mice with dengue structural proteins and nonstructural protein NS1 expressed by baculovirus recombinant induces resistance to dengue virus encephalitis. J Virol 62:3027–3031.